

Induction of the MexXY Efflux Pump in *Pseudomonas aeruginosa* Is Dependent on Drug-Ribosome Interaction

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MexXY is an inducible efflux system that contributes to the natural resistance of *Pseudomonas aeruginosa* to antibiotics. Experiments involving real-time PCR after reverse transcription in reference strain PAO1 showed concentration-dependent induction of gene *mexY* by various ribosome inhibitors (e.g., chloramphenicol, tetracycline, macrolides, and aminoglycosides) but not by antibiotics acting on other cellular targets (e.g., β -lactams, fluoroquinolones). Confirming a functional link between the efflux system and the translational machinery, ribosome protection by plasmid-encoded proteins TetO and ErmBP increased the resistance of a Δ *mexAB-oprM* mutant of PAO1 to tetracycline and erythromycin, respectively, as well as the concentrations of both drugs required to induce *mexY*. Furthermore, spontaneous mutations resulting in specific resistance to dihydrostreptomycin or spectinomycin also raised the minimal drug concentration for *mexXY* induction in strain PAO1. While strongly upregulated in a PAO1 mutant defective in gene *mexZ* (which codes for a putative repressor of operon *mexXY*), gene *mexY* remained inducible by agents such as tetracycline, chloramphenicol, and spectinomycin, suggesting additional regulatory loci for *mexXY*. Altogether, these data demonstrate physiological interplays between MexXY and the ribosome and are suggestive of an alternative function for MexXY beyond antibiotic efflux.

Pseudomonas aeruginosa is a nosocomial pathogen naturally recalcitrant to many antibiotics. Over the past decade, it has been realized that this poor drug susceptibility in part relies on the activity of two active efflux systems, namely MexAB-OprM and MexXY-OprM (1, 8, 15, 26). These saturable pumps, which are both able to accommodate an impressive variety of structurally unrelated molecules, tend to reduce the intracellular accumulation of antibiotics necessary for target inhibition. In *P. aeruginosa*, the very low permeability of the outer membrane porin channel makes the efflux process more efficient than in other gram-negative species such as *Escherichia coli* (18).

The Mex (for multiple efflux) pumps are three component systems composed of a transmembrane protein (MexB, MexY) belonging to the RND (for resistance nodulation, cell division) family of transporters, an outer membrane gated channel (OprM), and a periplasmic membrane fusion lipoprotein (MexA, MexX) presumed to play a role in pump assembly (16). Auxiliary OprM-like proteins such as OpmG and OpmI may also interact with MexX and MexY to form a tripartite functional pump (7). Like other members of the RND family, the MexB and MexY transporters use the proton motive force for exporting drugs in the external medium.

Despite their ability to expel numerous compounds out of the cell, MexAB-OprM and MexXY-OprM exhibit partially overlapping substrate profiles. First recognized as a key mechanism in the natural antimicrobial resistance of *P. aeruginosa*, the MexAB-OprM system is constitutively produced under standard laboratory conditions and affords protection against a

wide array of antibiotics, including tetracycline, chloramphenicol, quinolones, trimethoprim, and β -lactams (imipenem excepted) (13). On the other hand, MexXY-OprM contributes to the low intrinsic susceptibility of the microorganism to a number of substrates such as aminoglycosides, tetracycline, and erythromycin, which induce the production of proteins MexX and MexY encoded by the *mexXY* operon (1, 12). Interestingly, it has been reported that another MexXY substrate, ofloxacin, was unable to upregulate MexXY production, suggesting that only ribosome inhibitors might do so, at least in wild-type cells expressing the MexAB-OprM system (12).

To date, no information has been available on the regulation of *mexXY* expression, except that mutations occurring in the divergently transcribed repressor gene *mexZ* result in MexXY overproduction and moderate resistance to some of the pump substrates, including aminoglycosides (MIC increased two- to eightfold) (25, 26). These mutants (type AgrZ), which are frequently isolated from the sputa of cystic fibrosis patients, are distinct from nonenzymatic (i.e., MexXY overexpressing) resistant isolates from other sources which generally harbor intact *mexZ* genes (type AgrW) (10, 23). This strongly suggests that like *mexAB-oprM* (3, 21), *mexXY* is under the control of several regulatory loci. Recently, the *mexZ*-encoded product, a probable TetR family repressor, has been shown to bind as a dimer to the *mexZ-mexXY* intergenic region encompassing two putative overlapping promoters set in opposite orientations (14).

The present study examines the antibiotic-induced expression of *mexXY* and highlights a functional connection between the ribosome and this efflux system.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The strains and plasmids used in this study are listed in Table 1. Bacteria were grown at 37°C on Mueller-Hinton agar plates (Bio-Rad, Ivry sur Seine, France) or in Mueller-Hinton broth

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or phenotype	Source or reference
<i>P. aeruginosa</i> strains		
PAO1	Wild-type susceptible strain	
Mut-Gr1	<i>mexZ</i> -deficient mutant of PAO1 overexpressing operon <i>mexXY</i> ^a	25
KJ41	Dihydrostreptomycin-resistant mutant of PAO1	This study
KJ42	<i>rpsE</i> mutant of PAO1 resistant to spectinomycin	This study
K1119	PAO1 Δ <i>mexAB-oprM</i>	9
FB1	PAO1 Δ <i>mexB</i>	F. El Garch (unpublished)
Plasmids		
pMMB206	Broad-host-range cloning vector; Cm ^r	17
pXZL1082	pMMB206 carrying <i>tetO</i> ; Cm ^r Tet ^r	This study
pMLS001	pMMB206 carrying <i>ermBP</i> ; Cm ^r Ery ^r	This study

^a Nonsense mutation (underlined) of Mut-Gr1 at position 307 of repressor gene *mexZ* (CAG→stop codon TAG).

(MHB; Becton Dickinson Microbiology Systems, Cockeysville, Md.) with adjusted concentrations of divalent cations Ca²⁺ and Mg²⁺. Mutant K1119 was cultivated in the presence of 30 µg per ml chloramphenicol to maintain the pMMB206-based plasmid constructs.

Drug susceptibility tests. MICs were determined by the conventional twofold broth microdilution technique in MHB with inocula of approximately 5 × 10⁴ bacteria per ml (2). The antibiotics tested were obtained from Sigma-Aldrich (tetracycline, spectinomycin, ofloxacin, dihydrostreptomycin), Schering-Plough (gentamicin), Abbott Laboratories (erythromycin, vancomycin), Bristol Myers Squibb (cefepime), Bayer Pharma SA (ciprofloxacin), Merck (chloramphenicol), GlaxoSmithKline (ticarcillin), and Pfizer (azithromycin).

Isolation of drug-resistant mutants of *P. aeruginosa* PAO1. *P. aeruginosa* mutants KJ41 and KJ42 were selected on Mueller-Hinton agar medium supplemented with 500 µg per ml dihydrostreptomycin and 1,024 µg per ml spectinomycin, respectively. Compared with wild-type parent PAO1, KJ42 harbored a mutation in gene *rpsE* leading to a Val₂₂→Leu substitution in ribosomal protein S5, an alteration known to confer specific resistance to spectinomycin (27) (data not shown). In contrast, sequencing of gene *rpsL* in KJ41 did not show any mutations that could account for the resistance of this mutant to dihydrostreptomycin, an aminoglycoside closely related to streptomycin (5; data not shown).

Cloning of *ermBP* and *tetO* genes. The *ermBP* methylase gene of plasmid pJIR229 (22) was amplified on a 737-bp fragment via PCR using primers *ermBP*-F (5'-GGATCCGGATCCAGAAGGAGTGATTACATGAAC-3'; tandem BamHI sites underlined) and *ermBP*-R (5'-AAGCTTAAGCTTTAGAAT TATTTCTCCCGTTA-3'; tandem HindIII sites underlined). The formulation of reaction mixtures was as described previously (23), except for the absence of dimethyl sulfoxide, and amplification of *ermBP* was achieved by heating at 95°C for 30 s followed by 25 cycles of 95°C for 30 s, 47°C for 30 s, and 72°C for 1 min and finishing with 72°C for 7 min. Once purified as described before (23) and digested with BamHI and HindIII, the PCR product was subsequently cloned into BamHI-HindIII-restricted plasmid vector pMMB206 to yield pMLS001. Subsequent nucleotide sequencing confirmed that no mutation had been engineered into *ermBP* during PCR. The *tetO* gene of plasmid pUOA2 (11) was excised on a HincII fragment and cloned into SmaI-restricted pMMB206 to yield pXZL1082.

Quantitative real-time RT-PCR. Overnight cultures of strains PAO1, KJ41, KJ42, Mut-Gr1, K1119(pMMB206), K1119(pXZL1082), K1119(pMLS001), or FB1 in MHB were diluted 1:100 into fresh medium containing subinhibitory concentrations of tetracycline (0.06 to 8 µg per ml), chloramphenicol (1 to 64 µg per ml), gentamicin (0.06 to 0.5 µg per ml), dihydrostreptomycin (0.5 to 128 µg per ml), spectinomycin (8 to 2048 µg per ml), erythromycin (2 to 256 µg per ml), azithromycin (0.25 to 64 µg per ml), ofloxacin (0.5 µg per ml), ciprofloxacin (0.06 or 0.12 µg per ml), vancomycin (128 and 512 µg per ml), cefepime (1 and 2 µg per ml), or ticarcillin (8 µg per ml) and incubated with vigorous shaking at 37°C for 4 h (induction phase). Total RNA extraction and reverse transcription (RT) were performed as previously reported (10). The *mexY* cDNA was quantified in a Rotor Gene RG3000 Real Time PCR machine (Corbett Research, Sydney, Australia) in the presence of SybrGreen (QIAGEN), with primers *MexY*1A (5'-TTACCTCCAGCGGC-3') and *MexY*1B (5'-GTGAGCGCGGCGTTGTG-3'). Expression of the housekeeping gene *uvrD* was assessed in parallel with the primer pair *UvrD*1 (5'-CAGCCTCGCCCTACAGCA-3') and *UvrD*2 (5'-GGATCTGGAAGTTCTCGCTCAGC-3') (20). Confirming other findings (7), preliminary experiments demonstrated that transcription of *uvrD* was fairly stable in

bacteria exposed to antibiotics even at relatively high concentrations (e.g., half the MIC) (data not shown). Overnight incubation of strain K1119 in MHB containing 30 µg per ml chloramphenicol for selective maintenance of vector pMMB206 and its derivatives had no significant effect on *mexY* expression despite inducing activity of this antibiotic because chloramphenicol was inactivated by the vector-encoded chloramphenicol acetyltransferase enzyme (data not shown).

RESULTS AND DISCUSSION

Expression of *mexXY* in drug-exposed *P. aeruginosa* PAO1.

Production of MexX, the periplasmic lipoprotein which presumably interacts with transporter MexY and outer membrane protein OprM to form a functional tripartite efflux system (MexXY-OprM), was shown to increase in *P. aeruginosa* cells exposed overnight to subinhibitory concentrations of tetracycline, erythromycin, or gentamicin (12). Because MexXY expression gets higher at the onset of the stationary phase (C. Vagne, unpublished), we measured the amounts of *mexY* transcripts (as a measure of *mexXY* expression) by RT-PCR in wild-type strain PAO1 exposed for 4 h to various concentrations of drugs acting on different cellular targets. Under these conditions, the bacterial cells expressed *mexY* at very different levels depending on the nature and concentration of the antibiotic used (Fig. 1). Only ribosome inhibitors among the 13 agents tested were able to induce *mexY* expression significantly (at least twofold) compared with untreated bacteria. A first group of compounds, including chloramphenicol, spectinomycin, tetracycline, and azithromycin, was found to activate *mexY* expression at very low concentrations, which did not impact bacterial growth (e.g., 1/64 MIC). A second group of compounds including gentamicin, erythromycin, and dihydrostreptomycin displayed much lower inducing activities on *mexY*, increasing the gene transcription levels at growth-inhibitory concentrations only (i.e., 1/4 or 1/2 of the MIC). In contrast to the results reported by Masuda et al. (12) and despite repeated attempts, ofloxacin and ciprofloxacin (and also cefepime) even at 1/2 MIC were unable to elicit *mexY* expression in *P. aeruginosa* strain FB1, a *mexB*-null mutant that still produces OprM (data not shown). Since all the *mexXY* inducers identified in these experiments have in common the ability to inhibit protein synthesis, we postulated that the MexXY efflux system might directly or indirectly be induced as a result of the interaction of these inhibitors with the ribosome.

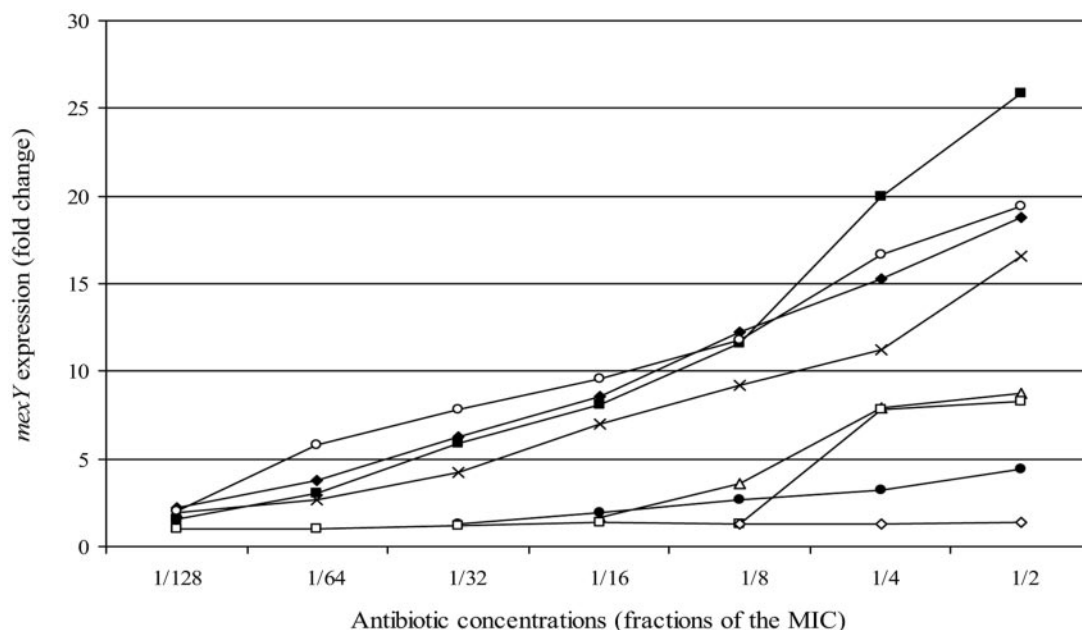


FIG. 1. Drug-inducible *mexY* expression in *P. aeruginosa* PAO1. Quantitative RT-PCR of *mexY* mRNA levels of strain PAO1 exposed to subinhibitory concentrations of chloramphenicol (solid squares), spectinomycin (open circles), tetracycline (solid diamonds), azithromycin (crosses), gentamicin (open triangles), erythromycin (open squares), dihydrostreptomycin (solid circles), or cefepime (open diamonds). The data are mean values of three independent experiments and indicate fold change relative to PAO1 untreated. The results with ciprofloxacin, ofloxacin, vancomycin, and ticarcillin were identical to that with cefepime (not shown). Two micrograms of total RNA was reverse transcribed to cDNA, and then *mexY* transcripts were quantified by RT-PCR and normalized to *uvrD* levels as described previously (10).

Ribosomal protection compromises drug inducibility of *mexXY*. The above results did not rule out the possibility that some drugs might interact directly with MexZ and modulate its repressor activity, reminiscent of tetracycline induction of the *tetA(B)* efflux determinant following its interaction with and modulation of the activity of the TetR repressor of *tetA(B)* gene expression (6). Still, a recent study showing that drugs known to induce *mexXY* expression neither bind to MexZ nor have any impact on MexZ binding to the *mexXY* promoter region (14) precludes this possibility. To assess the possible significance of antibiotic-ribosome interaction with respect to antibiotic induction of *mexXY* expression, then, the impact of known ribosomal protection mechanisms (*tetO*, tetracycline resistance; *ermBP*, erythromycin resistance) on antibiotic-inducible *mexXY* expression was examined in the *P. aeruginosa* Δ *mexAB-oprM* mutant K1119. Strain K1119 was used to prevent the MexAB-OprM-mediated baseline efflux of tetracycline and erythromycin that could have masked the impact of TetO and ErmBP proteins on resistance and *mexY* induction (12). Protein TetO expressed from plasmid pXZL1082 increased the tetracycline MIC 16-fold (from 2 to 32 μ g per ml) in K1119 (compared with vector pMMB206), while production of protein ErmBP from plasmid pMLS001 resulted in stronger resistance to erythromycin in this mutant (from 64 to >2,048 μ g per ml). Confirming our starting hypothesis, and in parallel with these higher MICs, ribosomal protection with proteins TetO and ErmBP increased the minimal concentrations of tetracycline (from 0.06 to 1 μ g per ml, Fig. 2A) and erythromycin (from 4 to 64 μ g per ml, Fig. 2B), respectively, required to activate *mexY* expression in strain K1119. These data clearly

indicate that *mexXY* transcription is induced as a result of ribosome inhibition or alteration by the tested antibiotics.

Spontaneous aminoglycoside-resistant mutants are compromised for drug induction of *mexXY*. A spontaneous mutant of *P. aeruginosa* strain PAO1 selected on spectinomycin was also used to assess the effect of “ribosomal protection” on *mexXY* antibiotic inducibility. This mutant, named KJ42, was found to harbor a single amino acid change in ribosomal protein RpsE and to be much less susceptible to spectinomycin than its wild-type parent (MIC increased from 512 to >2,048 μ g per ml). As for TetO and ErmBP, the mutation in KJ42 substantially increased the minimal inducing concentration (i.e., the lowest concentration of drug still capable of inducing *mexXY* expression) of spectinomycin (from 8 to 128 μ g per ml; Fig. 2C). A second spontaneous mutant of PAO1, dubbed KJ41, selected on and exhibiting specific resistance to dihydrostreptomycin (MIC increased from 16 to >256 μ g per ml) but with no apparent mutations in the *rpsL* gene also showed a marked increase in the minimal concentration of, in this case dihydrostreptomycin, needed to induce *mexXY* (from 2 to 64 μ g per ml, Fig. 2D). These data are consistent with mutations impacting drug-ribosome interactions also compromising *mexXY* induction by those same drugs.

RelA-independent induction of MexXY. An attractive hypothesis would be that signal molecules directly or indirectly turn on *mexXY* expression when the ribosome is functionally impaired. A guanine nucleotide derivative of GDP, (p)ppGpp, is synthesized in abundance by ribosome-associated enzyme RelA during the stringent response when bacterial cells are starved for amino acids (4). Since accumulation of (p)ppGpp is

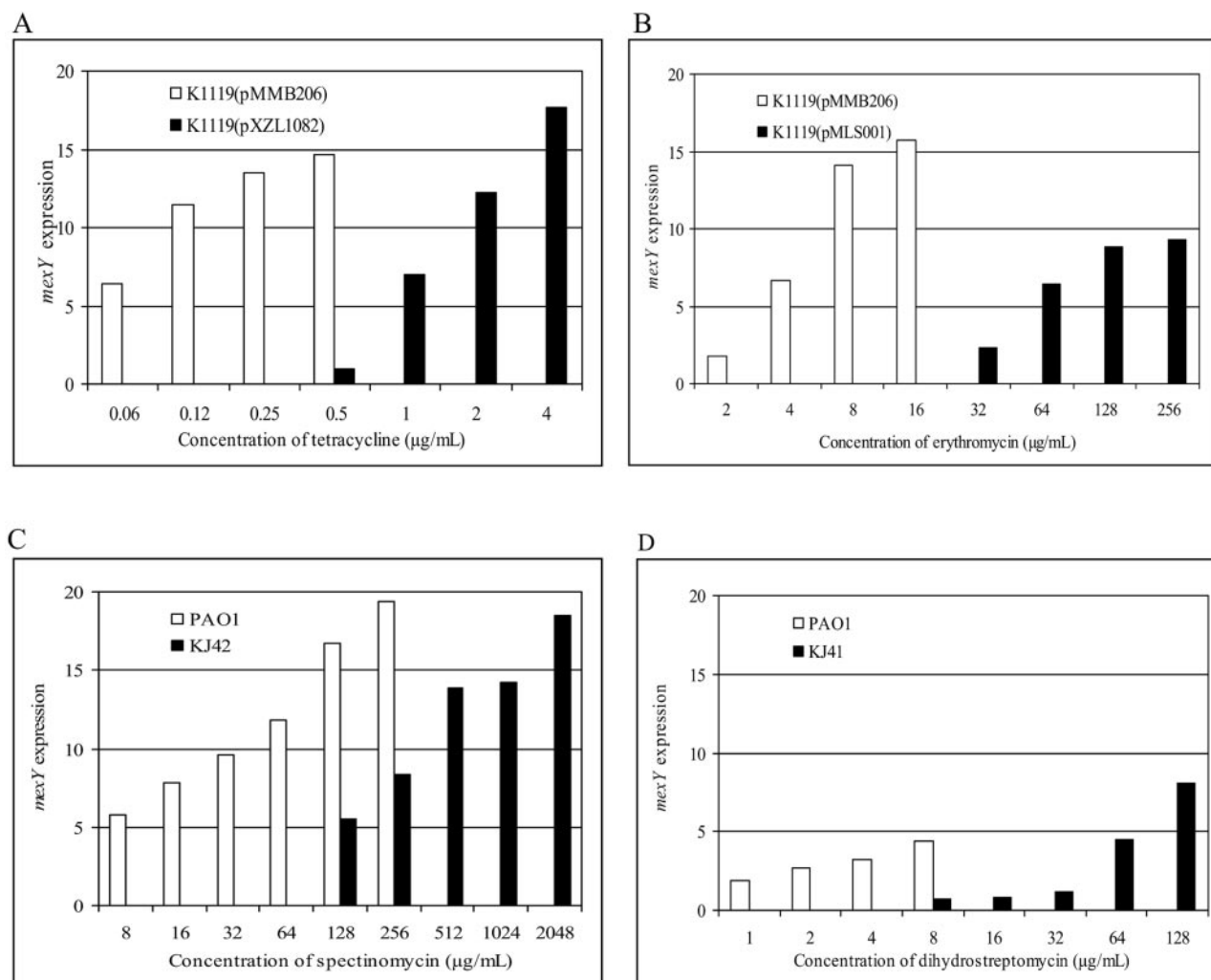


FIG. 2. Effect of ribosomal protection and spontaneous mutations on drug-induced expression of *mexY* in *P. aeruginosa* strains PAO1 and K1119. The indicated *P. aeruginosa* strains were cultured in the presence of the indicated antibiotics, and *mexY* expression was assessed using RT-PCR as for Fig. 1. (A) *P. aeruginosa* K1119 carrying pMMB206 or pXZL1082 (pMMB206::tetO) exposed to tetracycline; (B) *P. aeruginosa* K1119 carrying pMMB206 or pMLS001 (pMMB206::ermBP) exposed to erythromycin; (C) PAO1 and its spectinomycin-resistant mutant KJ42 exposed to spectinomycin; (D) PAO1 and its dihydrostreptomycin-resistant mutant KJ41 exposed to dihydrostreptomycin.

associated with pleiotropic effects on cell physiology (4), we looked at the influence of *relA* inactivation on the resistance pattern of *P. aeruginosa* PAO1 to various MexXY inducers, including aminoglycosides and tetracycline. However, no change in susceptibility was observed between the mutant (24) and its wild-type parent, suggesting that RelA is not involved in the induction of the efflux system (data not shown).

Role of MexZ in drug inducibility of *mexXY*. While antibiotic induction of *mexXY* expression cannot be explained on the basis of these agents modulating MexZ repressor activity directly, indirect involvement of MexZ in the induction of *mexXY* as a result of a series of events that follows drug ribosome interaction cannot be ruled out. For example, some ribosomal constituents or products produced by the cell, in response to drug-ribosome interaction could bind MexZ and modulate its repressor activity. To assess then the need for MexZ to mediate the drug inducibility of *mexXY* expression, the impact of *mexZ* loss (in mutant strain Mut-Gr1) on *mexXY* expression

with/without antibiotic exposure (at 1/2 MIC) was examined. As indicated in Fig. 3, wild-type strain PAO1 exposed to tetracycline showed a substantial (20-fold) increase in *mexXY* expression with loss of *mexZ* providing the same increase in expression even without tetracycline exposure, consistent with MexZ mediating the tetracycline induction of *mexXY*. Chloramphenicol- and spectinomycin-treated (at 1/2 MIC) PAO1 also showed *mexXY* expression at levels comparable to that seen in untreated Mut-Gr1 (compare Fig. 1 and 3) consistent with MexZ mediating their induction of *mexXY* as well. Exposure of Mut-Gr1 to tetracycline (or spectinomycin or chloramphenicol) did, however, provide for a very modest (<2-fold) increase in *mexXY* expression, suggesting that there may be additional mediators of drug-inducible *mexXY* expression in *P. aeruginosa*. The occurrence of additional regulator genes for *mexXY* has already been suggested as MexXY-overproducing *P. aeruginosa* strains isolated from non-cystic fibrosis patients often exhibit intact *mexZ* genes (10, 23, 25). These mutants of

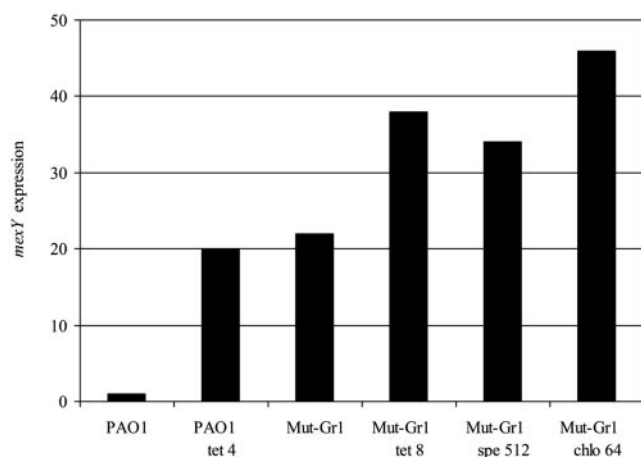


FIG. 3. Influence of *mexZ* mutation on drug-inducible *mexY* expression. Strain PAO1 and the *mexZ* mutant Mut-Gr1 were cultivated for 4 h in the presence of 1/2 MIC of tetracycline (Tet), spectinomycin (Spe), or chloramphenicol (Chlo), and *mexY* levels were measured using RT-PCR as in Fig. 1.

clinical origin have been named AgrW to differentiate them from the AgrZ mutants exhibiting alterations in gene *mexZ* (10). Whether these additional putative transcriptional regulators respond to intracellular signals generated by the injured ribosome itself or bind the inducing antibiotics remains unknown. It is important to note, however, that Mut-Gr1 produces a truncated MexZ that may possess residual repressor activity that responds to the presence of antibiotics (25).

Conclusion. Our results unambiguously demonstrate interplays between the MexXY-OprM efflux system and the translational machinery in *P. aeruginosa*. Further evidence for this is provided by the observation that mutants deficient in ribosomal protein RplA (26) or RplY (F. El Garch, manuscript in preparation) stably overproduce MexXY in drug-free culture medium. The observation that structurally unrelated antibiotics binding to different sites of the ribosome and interfering with different steps of protein synthesis are all able to induce MexXY production also supports the notion that the pump is activated when the ribosome is functionally impaired, regardless of the nature of the inhibition. While bacterial pumps have been the topic of extensive research over the past decade, limited information is still available on the precise physiological functions of these transporters. It is interesting to note that one of the best known efflux systems, namely Blt in *Bacillus subtilis*, is involved in the outward transport of spermidine, a polyamine which interacts with the ribosome and which modulates the activity of protein synthesis inhibitors (19, 28, 29). The data provided by this study shed some light on the role of an RND pump. Identification of the natural substrates transported by MexXY-OprM in *P. aeruginosa* would provide useful insights into the intended function of this efflux system and its connection to the ribosome.

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